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(54) Title: TRANSGENIC ANIMAL EXPRESSING ALZHEIMER'S TAU PROTEIN

(57) Abstract: The present invention provides transgenic non-human animals and non-human mammalian somatic and germ cells harbouring a human DNA sequence encoding Alzheimer's Disease (AD) derived tau protein, capable of inducing AD pathology in transgenic animals. Alzheimer's tau protein is expressed on specific genetic backgrounds allowing also simulation of different human diseases including hypertension, diabetes, hyper-cholesterolemia, which are associated with neurodegeneration and are considerable risk factors for AD development. Transgenic animals and cells of the present invention exhibit neurofibrillary pathology and may serve as in vivo and also in vitro assay systems for screening and developing therapeutic and preventive substances and also diagnostic markers and probes for tauopathies and AD. Furthermore these transgenic animals and cell lines derived thereof provide an in vivo and in vitro assay system for neurodegenerative disorders preferably tauopathies and AD which are the result of combinations with other disease as hypertension and others representing risk factors associated with the process of neurodegeneration.

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Transgenic Animal Expressing Alzheimer's Tau Protein

Neurodegenerative diseases represent a heterogeneous group of genetic and acquired neurological disorders that result in severe and progressive cognitive and motor impairment with onset during mid- to late-life (1). The most common cause of dementia is Alzheimer's disease. In less than 5% of the cases Alzheimer's disease genetic factors are involved, the rest of cases are sporadic.

Tau protein is the protein expressed in central nervous system and plays a critical role in the neuronal architecture by stabilizing intracellular microtubule network. Impairment of the physiological role of the tau protein either by truncation, hyperphosphorylation or by disturbing the balance between the six naturally occurring tau isoforms leads to the formation of neurofibrillary tangles (NFT), dystrophic neurites and neuropil threads. These structures represent ultrastructural hallmarks of Alzheimer's disease. The major protein subunit of these structures is microtubule associated protein tau (2,3). The amount of NFT found in autopsies of AD patients correlates with clinical symptoms including intellectual decline. Therefore tau protein plays a critical role in AD pathology. The recent discovery of cosegregation of specific mutations in the tau gene with the disease frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) has confirmed that certain abnormalities in the tau protein can be a primary cause of neurodegeneration and dementia in affected individuals (4,5). However there is no experimental model that would directly address the details on the role of tau protein in pathogenesis of AD.

Recent transgenic models of AD are based on expression of proteins associated with FAD (APP, PS1, ApoE etc.) either mutated or not. They include single and multigenic humanized animals. However none of the models recapitulates both hallmarks of AD, they usually do not show neurofibrillary pathology and none of them includes the possibility of investigation the function of environmental risk factors, which play a major role in the development of AD.

Currently available animal models showing some neurofibrillary changes are based solely on transgenic expression of mutated tau derived from frontotemporal dementia linked to chromosome 17 (FTDP17). Typical example of this group is JNPL3 transgenic mouse (6). Another evidence on the role of mutated tau have been published recently by Goedert and Spillantini (7).

In another transgenic animal model an overexpression of four repeat tau protein was investigated. Prominent axonopathy in the brain and spinal cord was reported and it was shown that an overexpression of four repeat tau protein isoform is sufficient to alter physiology of neurons. In the model, axonal dilations with accumulation of neurofilaments were documented in the neurons of central nervous system however without formation, however of intraneuronal neurofibrillary tangles (8).

The transgenic mouse was also produced in order to examine the normal cellular function of tau and its role in pathogenesis. The transgenic mouse overexpress a genomic tau transgene and also tau cDNA transgene. The comparison of two models shows that the distribution of tau is similar in both transgenic lines. Tau-immunoreactive axonal swellings were found in the spinal cords of the cDNA mice, which correlated with a hind-limb abnormality, whereas neuropathology was not observed in the genomic mice (9).

Despite of the advantage of rat as an experimental model in neurobiology in comparison to mice, it is surprising that no tau transgenic model for neurofibrillary pathology was produced. In general it is considerable lower amount of transgenic rat models of neurodegeneration produced till now. None of them recapitulates tau pathology, which is typical for AD.

Czech C, et al. (12) described a transgenic rat model which comprises of mutations in the gene coded for presenilin 1 (PS1). PS1 is considered as a cause for the majority of cases of early onset familial Alzheimer's disease, however the published model did not show sufficient signs of AD associated pathology. Echeverria V and Cuello AC. (13) comment on the role of intra-

cellular accumulation of beta amyloid and the possible neuropathological role of intracellular amyloid-beta accumulation in Alzheimer's disease. Their present recent observations from a rat transgenic model with a phenotype of intracellular accumulation of A-beta fragments in neurons of the hippocampus and cortex, however without plaque and NFT formation. Therefore for rats, which is the second most often used animal in transgenic studies applies the same as for mice: there is no transgenic rat described in scientific literature that would express truncated tau protein and showed NFT formation.

Till now there is no experimental model available for investigation of etiology of AD in a connection with hypertension or other risk factors like diabetes and dislipidemia, which are associated with AD. Genetic background of experimental transgenic animals plays a role in the development of appropriate transgenic model

It is well known that AD is associated with certain environmental risk factors. It was shown in epidemiological studies, that risk factors for vascular disease and stroke are associated with cognitive impairment and Alzheimer's disease; moreover the presence of cerebrovascular disease intensifies the presence and severity of the clinical symptoms of Alzheimer's disease (14). Another link between hypertension and dementia of AD type is indicated in a Rotterdam Study (15).

Therefore it is important to develop an experimental model for AD, which would mimic the main pathological features of the disease and simultaneously allows an expression of environmental factors associated with AD etiology.

It is therefore an object of present invention to provide an experimental model, which reflects the hallmarks of AD as close as possible and also the assay system for substances for treatment, prevention and diagnosis of AD.

The present invention relates to a transgenic non-human animal expressing cDNA encoded Alzheimer's tau protein in the brain of the transgenic animals. These animals exhibit NF pathological

activity in brains. The invention further relates to a transgenic animal, which can be used as a screening assay system in the process of identifying substances, which improve learning and memory, inhibit neurodegeneration and decrease the rate of neurofibrillary tangles (NFT) formation and their elimination from the brain. The animals can be used for identifying substances for prevention, treatment and diagnosis of neurodegenerative diseases, preferably tauopathies and AD. The transgenic animals can also be used for investigating the influence of AD associated risk factors, on the development of AD. Another use of animals lies in the process of drug target identification.

The present invention describes the engineering of transgenic animals producing AD changes induced by specific transgene, resembling Alzheimer's disease in these animals. This provides an investigative tool to the discovery and development of drugs and diagnosis and to further study the role of normal and diseased tau proteins in vivo.

The present invention relies on an induction of NF pathological activity in brain of animals solely by expressing of Alzheimer's tau protein in the brains of transgenic animals. Hallmarks of AD are induced in animals, which are prone to induction of risk factors associated with etiology of AD, which is another advantage of the present invention.

All technical and scientific terms used in the description of preferred embodiments have - unless specified otherwise - the same meaning as commonly understood in the field of art to which this invention belongs. The preferred methods, the laboratory procedures in cell culture, molecular genetics, biochemistry, and nucleic acid chemistry and materials are described in the invention, but any methods and materials similar or equivalent can be used in testing of the present invention. For procedures like recombinant nucleic acid preparation, biochemical analysis, cell culture, and transgene incorporation like electroporation, lipofection and microinjection standard techniques are used.

The term "Alzheimer's tau" refers to the group of specifically truncated isoforms of tau protein engineered genetically that

corresponds to those present exclusively in AD diseased brain. Alzheimer's tau is capable of inducing of NF activity on its own or in combination of other molecules in the brain. As used herein, the term Alzheimer's tau protein refers to the group of truncated tau forms present in AD-diseased human brain tissue.

The term "transgene" is used herein to describe genetic material, which has been artificially inserted into the genome of a cell, particularly a mammalian cell for implantation into a living animal. For specific countries, it may be necessary to specifically exclude certain subject matter from this aspect, such as human totipotent stem cells as such or processes which lead to such cells, falling under Art.6 (1) and (2) of the EC Directive 98/44/EC.

By "Alzheimer's disease" (abbreviated herein as "AD") is meant a condition associated with formation of neurofibrillary tangles and beta amyloid plaques, as well as impairment in both learning and memory. "AD" as used herein is meant to encompass both AD as well as AD-type pathologies, i.e., diseases of the central nervous system with symptoms similar to AD.

By "Neurofibrillary activity" (abbreviated herein as "NF" activity) is meant a condition associated with formation of neurofibrillary tangles in central nervous system with symptoms similar to AD.

By "symptoms similar to AD" and "phenomenon associated with AD" is meant a structural, molecular, or functional event associated with AD, particularly such an event that is readily assessable in an animal model. Such events include, but are not limited to, neurofibrillary pathology, learning and memory deficits, and other AD-associated characteristics.

By "transgenic animal" is meant a non-human animal, usually a mammal (e.g., a mouse, rat, rabbit, hamster, etc.), having a non-endogenous (i.e., heterologous) nucleic acid sequence present as an extrachromosomal element in a portion of its cell or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic

acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos of the host animal.

By "construct" is meant a recombinant nucleic acid sequence, generally recombinant DNA sequences, operable linked to tissue specific or general promoter, which is generated for the purpose of the expression of a specific nucleotide sequence(s), in mammalian cells, or is to be used in the construction of other recombinant nucleotide sequences.

The present invention provides the structure, sequence and method for generating of DNA constructs used for transgenic animal preparation, which are characterised by the following features:

A DNA construct comprises a cDNA molecule coding for N- and C-terminally truncated tau molecules, wherein

- the molecules have truncated at least 30 nucleotides downstream of the start codon and truncated at least the 30 nucleotides upstream of the stop codon of the full length tau cDNA sequence coding for 4-repeat and 3-repeat tau protein, respectively, as given in Seq.accession number NM_173727 in GeneBank
- the minimally truncated tau core encompasses a protein fragment which is encoded by nucleotides nos 744 - 930 (seq ID No. 9) (numbered according to tau protein isoform 43)
- said DNA constructs are coding for proteins, which have neurofibrillary (NF) pathology producing activity when expressed in brain cells of animals.

This NF pathology producing activity can be quantified by counts of neurofibrillary tangles per selected brain region, e.g. using stereology approach. Alternatively, neurobehavioural tests can be used for reliable evaluation of cognitive scores in learning memory-addressing tasks.

Specifically preferred tau cDNA molecules according to the present invention comprise a nucleotide sequence selected from the group of SEQ ID NOS 1 to 14. The sequences are depicted in Fig. 1.

In a preferred embodiment of the invention, said cDNA molecules represent a group of N- and C-terminally double truncated cDNAs, which comprises the following nucleotide sequences:

(Derivatives from 4-repeat tau (tau 43) are labeled R4 and derivatives from 3-repeat tau (tau 43) are labeled R3). Numbering used here is derived from full length tau cDNA described in Goedert et al.(24):

SEQ ID NO: 1 (91-1059, R4)

ATGCACCAAGACCAAGAGGGTGACACGGACGCTGGCCTGAAAGCTGAAGAAGCAGGCATTG-
GAGACACCCCCAGCCTGGAAGACGAAGCTGCTGGTCACGTGACCCAAGCTCGCATGGT-
CAGTAAAAGCAAAGACGGGACTGGAAGCGATGACAAAAAAGCCAAGGGGGGCTGATG-
GTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCCTCCAGGCCAGAAGGGCCAGGC-
CAACGCCACCAGGATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGT-
GAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCG-
GCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTG-
GCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGC-
CCCCGTGCCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCT-
GAAGCACCAGCCGGGAGGCGGGAAGGTGCAGATAATTAATAAGAAGCTGGATCT-
TAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCG-
GCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCT-
CATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAAATCTGAGAAGCT-
TGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTG-
GCGGAGGAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGCCAAAGC-
CAAGACAGACCACGGGGCGGAGATCGTGTACAAGTCGCCAGTGGTGTCTGGG-
GACACGTCTCCACGGCATCTCAGCAATGTC

SEQ ID NO: 2 (205-999, R4)

ATGGTCAGTAAAAGCAAAGACGGGACTGGAAGCGATGACAAAAAAGCCAAGGGGGGCTGATG-
GTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCCTCCAGGCCAGAAGGGCCAGGC-
CAACGCCACCAGGATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGT-
GAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCG-
GCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTG-
GCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGC-
CCCCGTGCCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCT-
GAAGCACCAGCCGGGAGGCGGGAAGGTGCAGATAATTAATAAGAAGCTGGATCT-
TAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCG-
GCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCT-
CATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAAATCTGAGAAGCT-

TGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTG-
GCGGAGGAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGCCAAAGC-
CAAGACAGACCACGGGGCGGAG

SEQ ID NO: 3 (277-999,R4)

ATCGCCACACCGCGGGGAGCAGCCCCTCCAGGCCAGAAGGGCCAGGCCAACGCCACCAG-
GATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGTGAACCTCCAAAAT-
CAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCGGCAGC-
CGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTGGCAGTG-
GTCCGTA CTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGCCCCCGTGC-
CCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCTGAAGCACCAGC-
CGGGAGGCGGGAAGGTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGTCCAGTCCAAGT-
GTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCGGCAGTGTGCAAATAGTCTACAAAC-
CAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAG-
GAGGTGGCCAGGTGGAAGTAAAATCTGAGAAGCTTGACTTCAAGGACA-
GAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTGGCGGAG-
GAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGCCAAAGCCAAGACAGAC-
CACGGGGCGGAG

SEQ ID NO: 4 (205-1089,R4)

ATGGTCAGTAAAAGCAAAGACGGGACTGGAAGCGATGACAAAAAAGCCAAGGGGGCTGATG-
GTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCTCCAGGCCAGAAGGGCCAGGC-
CAACGCCACCAGGATTCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGT-
GAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCG-
GCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTG-
GCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGC-
CCCCGTGCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCT-
GAAGCACCAGCCGGGAGGCGGGAAGGTGCAGATAATTAATAAGAAGCTGGATCT-
TAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCG-
GCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCT-
CATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAAATCTGAGAAGCT-
TGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTG-
GCGGAGGAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGCCAAAGC-
CAAGACAGACCACGGGGCGGAGATCGTGTACAAGTCGCCAGTGGTGTCTGGG-
GACACGTCTCCACGGCATCTCAGCAATGTCTCCTCCACCGGCAGCATCGACATGGTAGAC

SEQ ID NO: 5 (277-1089,R4)

ATCGCCACACCGCGGGGAGCAGCCCCTCCAGGCCAGAAGGGCCAGGCCAACGCCACCAG-
GATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGTGAACCTCCAAAAT-
CAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCGGCAGC-

CGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTGGCAGTG-
GTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGCCCCCGTGC-
CCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCTGAAGCACCAGC-
CGGGAGGCGGGAAGGTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGTCCAGTCCAAGT-
GTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCGGCAGTGTGCAAATAGTCTACAAAC-
CAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAG-
GAGGTGGCCAGGTGGAAGTAAATCTGAGAAGCTTGACTTCAAGGACA-
GAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTGGCGGAG-
GAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGCCAAAGCCAAGACAGAC-
CACGGGGCGGAGATCGTGTACAAGTCGCCAGTGGTGTCTGGGGACACGTCTCCACGGCATCT-
CAGCAATGTCTCCTCCACCGGCAGCATCGACATGGTAGAC

SEQ ID NO: 6 (715-999, R4)

ATCAAACACGTCCCGGGAGGCGGCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAG-
GTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTG-
GAAGTAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTG-
GACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCT-
GACCTTCCGCGAGAACGCCAAAGCCAAGACAGACCACGGGGCGGAG

SEQ ID NO: 7 (709-999, R4)

GATAATATCAAACACGTCCCGGGAGGCGGCAGTGTGCAAATAGTCTACAAACCAGTTGACCT-
GAGCAAGGTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGC-
CAGGTGGAAGTAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGG-
GTCCCTGGACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCT-
GACCTTCCGCGAGAACGCCAAAGCCAAGACAGACCACGGGGCGGAG

SEQ ID NO: 8 (715-954, R4)

ATCAAACACGTCCCGGGAGGCGGCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAG-
GTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTG-
GAAGTAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTG-
GACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCTG

SEQ ID NO: 9 (741-930, R4)

GTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCTCATTAG-
GCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAATCTGAGAAGCT-
TGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACCCACGTCCCTG-
GCGGAGGAAAT

SEQ ID NO: 10 (91-966, R3)

ATGCACCAAGACCAAGAGGGTGACACGGACGCTGGCCTGAAAGCTGAAGAAGCAGGCATTG-
GAGACACCCCCAGCCTGGAAGACGAAGCTGCTGGTCACGTGACCCAAGCTCGCATGGT-
CAGTAAAAGCAAAGACGGGACTGGAAGCGATGACAAAAAAGCCAAGGGGGGCTGATG-
GTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCCTCCAGGCCAGAAGGGCCAGGC-
CAACGCCACCAGGATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGT-
GAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCG-
GCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTG-
GCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGC-
CCCCGTGCCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCT-
GAAGCACCAGCCGGGAGGCGGGAAGGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGT-
GACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTG-
GAAGTAAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTG-
GACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCT-
GACCTTCCGCGAGAACGCCAAAGCCAAGACAGACCACGGGGCGGAGATCGTGTACAAGTCGC-
CAGTGGTGTCTGGGGACACGTCTCCACGGCATCTCAGCAATGTC

SEQ ID NO: 11 (205-906,R3)

ATGGTCAGTAAAAGCAAAGACGGGACTGGAAGCGATGACAAAAAAGCCAAGGGGGGCTGATG-
GTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCCTCCAGGCCAGAAGGGCCAGGC-
CAACGCCACCAGGATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGT-
GAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCG-
GCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTG-
GCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGC-
CCCCGTGCCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCT-
GAAGCACCAGCCGGGAGGCGGGAAGGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGT-
GACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTG-
GAAGTAAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTG-
GACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCT-
GACCTTCCGCGAGAACGCCAAAGCCAAGACAGACCACGGGGCGGAG

SEQ ID NO: 12 (277-906,R3)

ATCGCCACACCGCGGGGAGCAGCCCCCTCCAGGCCAGAAGGGCCAGGCCAACGCCACCAG-
GATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTGGTGAACCTCCAAAAT-
CAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCGGCAGC-
CGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTGGCAGTG-
GTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGCCCCCGTGC-
CCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCTGAAGCACCAGC-
CGGGAGGCGGGAAGGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGT-
GTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAAATCT-
GAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCAC-

CCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGC-
CAAAGCCAAGACAGACCACGGGGCGGAG

SEQ ID NO: 13 (205-996, R3)

ATGGTCAGTAAAAGCAAAGACGGGACTGGAAGCGATGACAAAAAGCCAAGGGGGCTGATG-
GTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCTCCAGGCCAGAAGGGCCAGGC-
CAACGCCACCAGGATTCCAGCAAAAACCCGCCCCGCTCCAAAGACACCACCAGCTCTGGT-
GAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCG-
GCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTG-
GCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGC-
CCCCGTGCCCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCT-
GAAGCACCAGCCGGGAGGCGGGAAGGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGT-
GACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTG-
GAAGTAAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCTGAAGATTGGGTCCCTG-
GACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCT-
GACCTTCCGCGAGAACGCCAAAGCCAAGACAGACCACGGGGCGGAGATCGTGTACAAGTCGC-
CAGTGGTGTCTGGGGACACGTCTCCACGGCATCTCAGCAATGTCTCCTCCACCG-
GCAGCATCGACATGGTAGAC

SEQ ID NO: 14 (277-996, R3)

ATCGCCACACCGCGGGGAGCAGCCCCTCCAGGCCAGAAGGGCCAGGCCAACGCCACCAG-
GATTCCAGCAAAAACCCGCCCCGCTCCAAAGACACCACCAGCTCTGGTGAACCTCCAAAAT-
CAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCGGCAGC-
CGCTCCCGCACCCCGTCCCTTCCAACCCACCCACCCGGGAGCCCAAGAAGGTGGCAGTG-
GTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGCCCCCGTGC-
CCATGCCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCTGAAGCACCAGC-
CGGGAGGCGGGAAGGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGT-
GTGGCTCATTAGGCAACATCCATCATAAACCAGGAGGTGGCCAGGTGGAAGTAAAATCT-
GAGAAGCTTGACTTCAAGGACAGAGTCCAGTCTGAAGATTGGGTCCCTGGACAATATCAC-
CCACGTCCCTGGCGGAGGAAATAAAAAGATTGAAACCCACAAGCTGACCTTCCGCGAGAACGC-
CAAAGCCAAGACAGACCACGGGGCGGAGATCGTGTACAAGTCGCCAGTGGTGTCTGGG-
GACACGTCTCCACGGCATCTCAGCAATGTCTCCTCCACCGGCAGCATCGACATGGTAGAC

The truncated tau sequences may be adapted due to the specific host cell whereto the construct is transferred (codon usage, codon preferences, homolog adaptations (exchanging one or more of the human codons with the host cell codon, if differences are present)).

Preferred features of the constructs:

The above cDNA sequences are linked to regulatory sequences in order to drive an expression of truncated tau protein in the brain. Modification of Thy-1 gene (25) was performed in such a way that regulatory sequence, which directs an expression into T-cells was deleted and truncated cDNA sequence was introduced in between Spe-I and Xho-restriction sites. The modified construct was stripped of prokaryotic sequences and introduced into male pronucleus via microinjection. Any functional promoters or promoter-enhancer complexes may be used for expression of above described truncated tau cDNAs.

According to another aspect, the present invention provides a method for the preparation and testing of molecules according to the present invention, characterized by the following steps:

- (a) construction of a recombinant prokaryotic cloning plasmids carrying coding sequences for a double truncated tau molecule with deletions covering at least the first first 30 and the last 30 nucleotides of three or four repeat tau cDNA molecules,
- (b) construction of an eukaryotic expression plasmid carrying appropriate promoters for brain expression or ubiquitous expression and respective cDNA sequences of truncated tau molecules and the combination thereof,
- (c) growing bacteria containing said plasmids and amplification, of the plasmids and their extraction in high quantity,
- (d) transfection of said gene constructs into COS-7 cells and their analysis using western blot technique,
- (e) isolation and purification of the gene construct such that all prokaryotic sequences are excluded, and dilution of transgenes in a buffer in concentration suitable for microinjection (see Example 1),
- (f) verification of gene constructs intended for microinjection.

This is performed by restriction analysis, gene sequencing and protein analysis after transient transfections into mammalian cells after which the correct size of proteins and their reactivity with specific monoclonal antibodies were evaluated (see Example 1, Fig. 2.b).

The present invention therefore provides a transgenic non-human animal of whose germ and/or somatic cells comprise the DNA construct encoding for Alzheimer's tau protein, which can be used as a suitable animal model of Alzheimer's disease.

It is therefore an object of the present invention to provide a transgenic non-human animal all of whose germ and/or somatic cells comprises the human truncated tau cDNA molecules. Cloned human Alzheimer's tau cDNA sequence is linked to a promoter sequence, which directs the expression of Alzheimer's tau in tissue specific manner or ubiquitously.

Transgenic animals of the invention are engineered using well known regular procedures of microinjection, into the male pronucleus of one day old fertile embryos of rats. The transgenes can be introduced into embryonic cells also by other methods known in the art, including transfection, lipofection, electroporation and with the help of retroviruses or by other means. Embryos carrying the transgene can then be implanted into pseudopregnant animals.

It is therefore an object of invention to provide a method for genotyping of newborn animals. PCR analysis using specific oligonucleotides that allow an amplification of target sequences of the transgene is therefore employed for this purpose. Oligonucleotides used for genotyping were designed in a way that the transcription initiation site of the transgene including the ATG codon and Kozak sequence could be verified eventually by DNA sequencing of the PCR product (primer pair A). Moreover one primer was anneals to the heterogeneous promoter and another primer anneals to nucleotide sequence coding for Alzheimer's tau protein. Similar designing criteria were applied for the stop codon (primer pair B). Integrity of the transgene therefore can be monitored this way. Sequences of the diagnostic oligonucleotides are therefore as follows:

Primer pair A:

Sense: 5'-GTGGATCTCAAGCCCTCAAG-3'

Antisense: 5'-GATCCCCTGATTTGGAGGT-3'

Expected size of PCR product is 235 nucleotides.

Primer pair B

Sense: 5'- AAGGTGACCTCCAAGTGTGG- 3'

Antisense: 5'- GGTATGCATGGAGGGAGAAG- 3'

Expected size of PCR product is 438 nucleotides.

Presence of the PCR fragment is therefore specific to animals with transgene integrated in the genome (see Example 2)

The founder animals can be used for breeding with wild-type animals to produce F1 generation of animals, which are heterozygous for the transgene. In further embodiments, these heterozygous animals can be interbred to obtain the viable transgenic embryos whose somatic and germ cells are homozygous for the genes coding for Alzheimer's tau protein.

Another object of the present invention is to provide non-human animals of which germ and somatic cells transiently or stably express said DNA construct coding for Alzheimer's tau protein and exhibiting NF pathology in the brain. The preferred animal of this invention is a rat, wherein the protein encoded by said DNA molecules is expressed in the brain.

The most preferred embodiment of the present invention is a transgenic non-human animal wherein the Alzheimer's tau protein encoded by said DNA molecules is expressed in the brain of the animal. The expression of Alzheimer's tau was detected by several monoclonal antibodies as described in Example 3 and documented on Fig. 3. Expression of the protein in different brain regions of transgenic lineage is documented on Fig. 4.

The present invention therefore provides a transgenic non-human animal wherein DNA molecules coding for Alzheimer's tau protein are stably integrated in the genome of said animals or otherwise present in the cell nucleus in order to be transcribed. Said animals are characterized by the following features:

- transgenes are transmitted to subsequent generations of animals according to Mendelian laws forming a transgenic lineage (see Example 4)
- the inherited transgene is expressing truncated proteins in

brains of the founder animals and the progeny as shown in Example 3 Fig. 4.

The present invention therefore provides the transgenic non-human animals, which exhibit neurofibrillary pathology in the central nervous system (CNS). Said animals are characterized by the following features:

- Expression of truncated tau proteins promotes the formation of neurofibrillary pathology (Example 5)
- PHF 1 immunoreactive NFTs showed that endogenous normal rat tau participate on the formation of NFTs (Example 5)
- Neurofibrillary tangles are present as intracellular inclusions and filaments in the neurons of CNS and are homologous to NFT in human AD diseased brains.
- Immunofluorescence revealed the presence of small - rod like structures similar to those observed in pre-clinical Alzheimer's disease - observed in human brain tissue (see Example 5).

In the preferred embodiment of the invention, transgenic animals display such neuropathology as neurofibrillary tangles, ghost tangles and neuropil threads that are formic acid sensitive. Neurofibrillary tangles are composed of an abnormal accumulation of intraneuronal filaments. The tangle components are comprised from Alzheimer's tau as well as normal tau molecules. Such transgenic animals serve as suitable model system for study of Alzheimer's disease and development of therapeutic, preventive and diagnostic substances.

The invention provides a transgenic animal developing NF pathology, and having a genetic background allowing induction of risk factors associated with AD, thereby representing a disease model for humans.

The present invention therefore provides a transgenic non-human animal, wherein the genetic background, in which DNA molecules are stably integrated in the genome of spontaneously hypertensive animal or otherwise present in the cell nucleus in order to be transcribed within the specific genetic background.

Another aspect of the invention is to provide an animal model comprising the combination of genetic factors and other risk factors associated with AD as follows:

- hypertension which is the most common risk factors of AD,
- diabetes which can be induced by diet (diabetes is important risk factor of AD),
- hypercholestroemia which can be induced by diet (another important risk factor of AD).

Said pathological situations can easily be induced in the provided animal model. Suitable examples were previously reported in literature (e.g. in 26, 27, 28).

Moreover the provided animal model is the first animal model, which expresses neurofibrillary pathology on the genetic background of SHR.

According to another aspect the present invention provides an animal, which represents an experimental model of sporadic Alzheimer's disease, said model permits an assessment of the role of risk factors associated with AD development. Risk factors play a critical role in the vast majority of AD cases, the model may therefore be considered as a model of sporadic AD which represents 90% of all cases of AD neurodegenerations.

In another preferred embodiment, the present invention provides a screening assay system for substances for the treatment prevention and diagnosis of Alzheimer's disease, which comprises:

- evaluation of substances by:
 - detecting changes of neurofibrillary pathology in said animals
 - measuring of neurobehavioural changes in transgenic animals
 - measuring of the cognitive score in transgenic animals
 - biochemical measuring of AD specific markers in animal tissues and body fluids
- a validation system for substances for the treatment and prevention of tauopathies preferably AD
- a validation system for the development of diagnostic markers and probes for the detection tauopathies, preferably AD

- a validation system for substances for treatment of hypertension, diabetes, dislipidaemia and hypercholesterolemia in combination with tauopathies preferably AD

The present invention therefore provides an in-vivo assay to assess the efficacy of substances, or therapies in particular NFT reducing or preventing therapies. The animals can be used for screening of substances or therapies for neurodegenerative diseases, in particular tauopathies, preferably AD and other neurodegenerative diseases accompanied by neurofibrillary tangles formation.

Therefore the present invention provides a model system consisting of transgenic animals, cells and assays that are useful in the study of the etiology and treatment of Alzheimer's disease. The assays are also useful for screening for substances that inhibit the formation of neurofibrillary pathology and other therapeutic effects of the substances.

The transgenic animals of the present invention and animal cells derived thereof are used for screening compounds for a potential effect in the treatment of Alzheimer's disease using standard methodologies. The compound is administered to the animals or introduced into the culture media over a period of time and in various dosages, then the animals or animal cells are examined for alterations in histopathology and Alzheimer's tau expression respectively. Furthermore, the ability to perform neurobehavioural testing to the transgenic animals enables cognitive function to be monitored following treatment with potential therapeutic agents. Screening of compounds leads to the selection of specific agents for the prevention or treatment or diagnosis of Alzheimer's disease. Many variations of screening methods are known to those skilled in the art and may be applied within the scope of the present invention.

The transgenic animal model of the present invention may also be used to unravel the molecular cascade of events leading to neurodegeneration and Alzheimer's disease such that therapeutic targets may be identified.

Still another aspect of the invention is to provide an in vitro assay based on cell lines derived from a transgenic animal or cell line derived from a transgenic rat embryo of the present invention, where the said assay is employed as a screening and validation tool for the discovery and development of Alzheimer's diagnosis and markers. Said in vitro assay is based on the cell lines and may also be employed as a screening and validation tool for the discovery of anti Alzheimer's therapeutic and preventive drugs.

The following examples are presented by the way of illustration and, because various other embodiments will be apparent to those in the art, the following is not to be construed as a limitation on the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1

Scheme of Alzheimer's tau protein cDNAs employed for engineering of gene expression constructs, which were used in production of transgenic animals of the present invention.

Fig. 2 Preparation and verification of gene constructs used for the production of transgenic animals. Construction of transgenic animals was performed via pronuclear microinjection of cloned gene constructs containing coding sequence for Alzheimer's tau. Gene constructs were verified by restriction analysis and PCR using transgene specific primers (panel A, agarose gel of PCR amplified and DNA fragment) as well as by DNA sequencing in order to control for potential mutations. Verification was followed by purification of gene constructs (panel B, agarose gel of pure transgene fragment) and transient transfections into mammalian cells (panel C, Western blot verification of protein expression). After the assays were completed the constructs were stripped of prokaryotic sequences and injected into one -day old rat embryos. The bottom flow chart shows a simplified procedure of transgenic animals production.

Fig. 3 Genotyping of transgenic founder animals and F1 generation of transgenic animals. Panel "A" shows controls for the

genotyping experiment (+C, positive control; NAC, no amplification control; NTC, no template control). Panel "B" shows results of PCR genotyping. Sample number 12 represents a transgene positive animal. Panel "C" outlines the breeding experiments performed to confirm founder animals. Furthermore the panel shows transgenic line of the present invention that has transmitted the transgene to the subsequent generations according to Mendelian laws.

Fig. 4

Expression of Alzheimer's tau protein in brain of transgenic rat. The left panel represents a Western blot analysis and right panel is presenting total amount of proteins loaded per lane. Alzheimer's tau protein migrates at the size of 35kD (lane 1, arrow), detected by pan-tau monoclonal antibody DC25. For Western blot an ECL technique was employed, and specific signal was observed after 1min exposure. The 7.5 ug of total protein extract from brain lysates of transgenic animal was loaded (lane 1) whereas 25 ug of proteins was loaded from wild type control animals. M, molecular size marker.

Fig. 5

Expression of Alzheimer's tau protein in different brain regions of transgenic rat. Western blot analysis of total protein lysates from transgenic rat brain regions. Lane 1, complete brain lysate of founder animal (generation F0) - 10ug proteins; lane 2, total brain lysate of transgenic animal of F2 generation; lanes 3-6, protein lysates from transgenic animal of F2 generation: 3 - hippocampus, 4 - subcortical area, 5 - cerebral cortex, 6 - spinal cord and medulla; lane 7- 8 represent brain lysates from control non-transgenic littermates of F2 generation: 7 - cortex, 8 - cerebellum (Total protein load per lanes 2-8 was 20ug).

Fig. 6

Silver staining of brain sections from transgenic animal and non-transgenic control wild type animal. Intracellular inclusions and neurofibrillary filaments were detected in the neurons of the CNS of transgenic rat, (panel B and C, transgenic animal). Wild type rats do not show these structures in the homologous

brain area (A, wild type control). Magnification: 200x (A and B), 400 x (C)

Fig. 7

Detection of neurofibrillary tangles using the pan-tau monoclonal antibody DC 25 in central nervous system of transgenic animal. mAb DC25 recognizes NFTs in the neurons of AD patients. The equivalent structures are recognized in the brain of transgenic rat (panel B and D). Wild type rats did not show any similar structures in the homologous brain area, (A, C). Magnification: 100x (A,B), 200x (C, D)

Fig. 8

Detection of neurofibrillary tangles by monoclonal antibody PHF-1 in central nervous system of the transgenic animal. PHF-1 is specific marker for tau phosphorylation. Immunoreactivity with this antibody shows that expression of Alzheimer's tau involves endogenous tau in the process of formation of NFTs. Furthermore, immunofluorescent staining shows small rod like structures similar to those observed in pre-clinical Alzheimer's disease brain tissues. Magnification: 100x (A), 200x (B), 1000x (C, D)

Fig. 9

Neuronal cell culture as a screening assay system for characterization of therapeutic leads and drug candidates. Primary cultures of transgenic and nontransgenic rat neurons can be used for this purpose after in vitro cultivation and effect of the drug candidates on the mitochondrial transport can be evaluated using videomicroscopy. Cortical (Cx) and hippocampal (Hipp) neurons loaded with vital dye (e.g. Mito-trek) are shown after 3 days in vitro cultivation (3DIV). Mitochondria (MT) are visualized.

Fig. 10

Histopathological features of Alzheimer's disease, recapitulated in transgenic animal of present invention. Presented neurofibrillary pathology is one of the major hallmark of Alzheimer's disease. Fig. 10 shows a comparison of neurofibrillary tangles detected by Gallyas silver technique (A, C) and also by immunohistochemistry (E) in AD diseased human brain, to

the equivalent pathological structures observed in transgenic animal of present invention. Immunochemical staining was performed with monoclonal antibody 7.51 which recognizes generic tau epitope in the tandem repeat region (29).

EXAMPLES

EXAMPLE 1: Design of gene constructs containing DNA sequence coding for Alzheimer's tau protein - human truncated tau gene; engineering and verification of gene constructs for ubiquitous and tissue specific expression in transgenic animals.

Construction of Alzheimer's tau expression vectors consist of procedures that include:

- design of coding sequence especially deletions and truncations are shown in 1.
- these portions of tau gene were PCR amplified with the help of proofreading DNA polymerase and oligonucleotides equipped with appropriate restriction sequences, so that they can be cloned under general or tissue specific promoters in an eukaryotic expression vector, cloning was performed using standard procedures in a common bacterial strains
- verification of cloned genes was performed in order to check directionality of the inserted gene which was confirmed by restriction digestions and or PCR analysis (Fig. 2A). In addition, the constructs were partially sequenced to exclude the possible mutated forms
- purification of fragment intended for microinjection (Fig. 2B)
- verification of protein expression using transfections of transgene into mammalian cells, mainly COS-7 and C6 rat glioma cells, and analysis of expression of proteins in a eukaryotic cells by western blot method (Fig. 2C).

All the methods are described in a manual: Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989 (14).

EXAMPLE 2: Production of transgenic rat

Cloned gene construct containing Alzheimer's tau coding sequence

was injected into male pronucleus of one day old embryos. Fertilized eggs were extracted from SHR donor females in separate experiments using routine techniques (15). After microinjecting of DNA, the zygotes were in vitro cultured in CO₂ incubator for about 12 hours and implanted into oviductus of pseudopregnant foster mothers. Founders were identified by PCR of transgene specific target sequence from genomic DNA. To determine the tau genotypes, genomic DNA was purified from about 0.5 cm of tail taken from each rat. PCR analysis, was used to identify offspring which contained the transgene. Oligonucleotides for diagnostic PCR screening were designed in such a way that integrity of the transgene could be easily determined. For determination of number of transgene copies integrated in rat genome a real time PCR analysis was used.

For example Tg line #318 contained 4 copies of the transgene, which was determined by real time PCR using Sybre Green method and BioRad PCR machine.

EXAMPLE 3: Genotyping of animals born after embryo implantation into foster mothers, identification of transgenic animals and assessment of transmission of the transgene to subsequent generations

DNA extracted from tail tips: Genomic DNA was extracted by DNeasy tissue kit, Qiagen.

Genotyping: Specific amplification of transgenes encoding double truncated tau forms was performed on genomic DNA derived from the parental generation of transgenic animals and is shown in Figure 3A and 3B. Further analysis of genomic DNA of the F1 generation revealed that transgenes are heritable since they were also identified in the offspring of parental generation. Transgenes encoding Alzheimer's tau are therefore fixed in chromosomal DNA of the animals. Genotyping was performed with two positive (plasmid and TG positive genomic DNA) and two negative controls (no amplification control heterologous genomic DNA; no template control for control of chemicals) as it is shown in Fig. 3A.

Analysis of transgene expression: Expression of mRNA derived

from the transgenes were assessed by RT-PCR analysis, applying generally known methods including RT-PCR and agarose gel electrophoresis. RNA was extracted from flash frozen tissue of transgenic animals and subjected to reverse transcription followed by specific amplification of the cDNA.

Rat breeding and generation of heterozygous and homozygous rats: Transgene positive rats were bred to wild-type SHR. Germline transmission was achieved in several lines. In this example Tg line #318 is shown. Male and female transgenic rat, each of which contained four copies of the truncated human tau protein gene were mated with each other and wild type SHR rat to expand transgenic colony. Mendelian splitting ratios of transgene in the offspring were observed (Fig. 3C).

The transgenic animals of the present invention can be used to cross breed with transgenic animals overexpressing the human APP containing familial Alzheimer's disease (FAD) mutation or PS1 (13). The resulting rat will express both APP and PS1 with or without FAD mutation on hypertensive genetic background. The effect of PS1 and APP on NFT formation can be studied both in vivo and in vitro. Specifically, the onset and severity of AD-related pathologies can be examined by means of immunohistochemistry.

EXAMPLE 4: Expression of Alzheimer's tau protein in the brain of transgenic rats

Protein expression was performed using western blot analysis employing tau protein specific monoclonal antibodies. We have analysed brain tissues of rats that were genetically positive for transgene and were able to transmit the transgene to subsequent generations. Experiments were controlled by simultaneous analysis of brain extracts from transgene negative age matched rats and transgene negative littermates.

Tissue sample preparation: approximately 2mg of freshly isolated brain tissue was mechanically homogenized in 30µl modified Hunt's lysis buffer (20mM Tris, pH 8.0, 100mM NaCl, 1mM EDTA, 0.5% Igepal CA630, 0.5% Triton X-100), which contained protease inhibit-

or mix Complete-EDTA free (Roche). 170 μ l of lysis buffer were added, homogenate was triturated with syringe and needle (22G) and frozen in liquid nitrogen. After slow thawing on ice, lysate was sonicated and centrifuged 15 minutes, 10000g at +4°C. Protein content in samples was determined by Bradford assay using Beckman spectrophotometer.

PAGE and Western blot. Proteins were separated on 12% denaturing polyacrylamid gel with 0.1% (w/V) SDS. Electrophoresis was performed in Tris-glycin buffer (25mM Tris, 192mM glycine, 0.1% SDS). Protein separation was followed by semi-dry blotting. Tris-glycin-isopropanol buffer system (25mM Tris, 192mM glycine, 20 % Isopropanol) was used. Tau proteins were stained with anti-tau monoclonal antibodies DC25 which recognize tau proteins equally as a mAb 7.51 (29) and also with mAb Tau-1 (21). The positive signal was determined using anti-mouse HRP antibody and ECL method. Efficiency of blotting procedure was controlled by Ponceau staining. Protein analysis has revealed the specific expression of Alzheimer's tau protein in total protein extract of brain of transgenic rat (Fig. 4) and also an expression of Alzheimer's tau protein in different brain regions of transgenic rat (Fig. 5).

EXAMPLE 5: Immunohistochemical analysis of brain of transgenic rat.

In order to perform immunohistochemical analysis of transgenic rat, several tau protein and Alzheimer specific monoclonal antibodies were used. Short description of monoclonal antibodies employed in these studies include PHF 1, which recognizes phosphorylated epitope Ser 396 - Ser 404; mAb DC 25 recognizes phosphate independent epitope Lys347 - Ile 353; mAb AT 8 recognizes phosphorylated epitope Ser 198 - Ser 202; mAb Tau 1 recognizes non-phosphorylated epitope, mAb Tau 5 recognizes phosphate independent epitope. (15, 16, 17, 18, 19, 20, 21, 22)

Light microscopy and immunofluorescence:

Tissues were prepared and sectioned as follows. Transgenic and control animals were anaesthetized with ether and immediately perfused intracardially with buffered 4 % paraformaldehyde,

brains were removed, cryoprotected with 20 % sucrose, quickly frozen and sectioned by cryostat at 50 μ m thickness. Some of the sections were shortly pretreated with 70 % formic acid prior staining procedure. Rat brain tissue sections were incubated with 1 % H_2O_2 in PBS and non-specific binding sites were blocked with incubation in blocking medium (PBS, 0, 1 % Triton, 5 % normal horse serum). The immunostaining was performed using antibodies listed above, then incubated with biotinylated horse anti-mouse antibody, followed by avidin-biotin complex and visualized with VIP and DAB solution. Tissue sections were mounted on gelatin-coated slides, dried, treated with graded alcohols, xylene and coverslipped with Entellan.

Silver staining:

Gallyas silver staining method that has been described previously (23) was adapted for free floating 50 μ m sections, with gold toning.

Immunofluorescent staining:

For indirect immunofluorescent labeling, sections were pretreated with 1 % $NaHBO_4$ for 30 min. to reduce brain tissue autofluorescence. Then stained with primary antibodies at 4 °C overnight, incubated with a horse anti mouse biotinylated antibody, followed by streptavidin-Alexa 488 conjugate.

Comparison of NF pathology as seen in the brains of patients suffering from AD with those observed in the brain of transgenic animal of present invention is represented in Fig. 10.

EXAMPLE 6: A screening assay systems and for drug leads and drug candidates for the treatment and prevention of Alzheimer's disease

The transgenic animals of the present invention can be used as a source of cells for cell culture. Both animals and cells can be used in assays of this invention. For example, cells of brain tissues expressing truncated tau gene can be cultured using standard culture techniques. The animals and cultured cells can be used as in vivo and in vitro systems for the study of the

role of drug candidates on cell architecture, cell division and apoptosis as well as a morphology of primary cultured neurons, sprouting of neurites, axon branching and axon elongation in primary cultures and also for investigation of organelle movement (Fig. 9). They can also be employed as a test system for neurotrophic effects of tested substances.

A study can be designed to screen for a quantitative or qualitative changes in an interaction of tau and tubulin and also in an interaction with organelle trafficking that can be modulated by tested substances.

A study can also be designed to screen for compounds that modulate the expression or activity of the cytoskeletal proteins in the process of building of synaptic connections.

A study can be also designed to screen for the compounds that can affect of NFT formation or protein-protein interaction, which could eventually lead to slowing down, inhibiting or elimination of NFT formation and this could be useful assay for screening for substances with potential of therapeutics for tauopathies and other neurodegenerative disease preferably AD. Effect of the compounds can be evaluated in cell cultures and also in vivo on the animals by means of neuropathological, neurophysiological and behavioural analysis. Many variations of screening methods are known to those of skill in the art and may be applied within the scope of the present invention.

These examples describe particular aspects and embodiments of the invention for illustrative purposes and are not limiting on the scope of the invention as listed in the claims below.

References

1. Martin, J.B., (1999) N. Engl. J. Med. 340, 1970-1980.
2. Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowther RA (1988a) Proc Natl Acad Sci USA 85: 4884-4888
3. Wischik CM, Novak M, Trogersen HC, Edwards PC, Runswick MJ, Jake R, Walker JE, Milstein C, Roth M, Klug A (1988b) Proc Natl Acad USA 85: 4506-4510
4. Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S., Houlden H, Pickering-Brown S, Chackraverty S, Isaacs A, Grover A (1998) Nature 393, 702-705
5. Spillantini NG, Murrell JR, Goedert M, Farlow MR, Klug A, Ghetti B (1998) Proc Natl Acad Sci USA 95, 7737-7741.
6. Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E. : Science 2001 Aug 24;293(5534):1487-1491
7. Goedert M, Spillantini MG.: Biochem Soc Symp 2001; 67: 59-71
8. Spittaels, K., C. Van den Haute, et al. : Am J Pathol (1999), 155 (6): 2153-65.
9. Duff, K., H. Knight, et al.: Neurobiol Dis (2000) 7 (2): 87-98.
10. Czech C, Lesort M, Tremp G, Terro F, Blanchard V, Schombert B, Carpentier N, Dreisler S, Bonici B, Takashima A, Moussaoui S, Hugon J, Pradier L.: Neuroscience (1998) Nov; 87 (2): 325-36
11. Echeverria V and Cuellar AC.: Mol Neurobiol (2002) Oct-Dec; 26(2-3):299-316
12. Breteler, MMB.: Neurobiology of Aging 21 (2000) 153-160

13. in't Veld B.A., A. Ruitenberga, A. Hofmana, B.H.Ch. Strickera, M.M.B. Bretelera,: Neurobiology of Aging 22 (2001) 407-412
14. Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989
15. Hogan,B. et al. Manipulating the Mouse Embryo, a Laboratory Manual. Cold Spring Harbor Laboratory, 1986
16. Bramblett GT, Goedert M, Jakes R, Merrick SE, Trojanowski JQ, Lee VM.: Neuron (1993) 10: 1089-99
17. Biernat J, Mandelkow EM, Schroter C, Lichtenberg-Kraag B, Steiner B, Berling B, Meyer H, Mercken M, Vandermeeren A, Goedert M, et al.: EMBO J (1992) Apr;11(4): 1593-7
18. Braak E, Braak H, Mandelkow EM: Acta Neuropathol (Berl). (1994); 87(6): 554-67.
19. Goedert M, Jakes R, Vanmechelen E.: Neurosci Lett (1995) Apr 21;189(3):167-9
20. Szendrei GI, Lee VM, Otvos L Jr.: J Neurosci Res (1993) Feb 1;34(2): 243-9
21. Binder LI, Frankfurter A, Rebhun LI. J: Cell Biol (1985) Oct;101(4):1371-8
22. LoPresti P, Szuchet S, Papasozomenos SC, Zinkowski RP, Binder LI.: Proc Natl Acad Sci U S A (1995) Oct 24;92(22):10369-73
23. Gallyas, F.: Acta Morphol.Acad.Sci.Hung. (1971) 19, 1-8.
24. Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989) Neuron 4, 519.
25. Gordon JW, Chesa PG, Nishimura H, Rettig WJ, Maccari JE, Endo T, Seravalli E, Seki T, Silver J.: Cell.1987 Jul 31; 50(3):445-52.

26. Damiano PF, Roson MI, Armando I, Nowicki S, Dascal E, Cuniberti L, Albornoz LE, de la Riva IJ. Potential role of glycerol leading to rat fructose hypertension. Hypertension 1999 Oct; 34 (4Pt 2):1007-11;.
27. Jackson B, Fabris B, Paxton D, Franze L, Johnston CI. High salt diet ameliorates effects of angiotension converting enzyme inhibition in spontaneously hypertensive streptozotocin diabetic rats. Clin Exp Pharmacol Physiol. 1990 Mar; 17(3); 229-34.
28. Turley SD, Hansen CT. Rates of sterol synthesis in the liver and extrahepatic tissues of the SHR/N-corpulent rat, an animal with hyperlipidemia and insulin-independent diabetes. J Lipid Res. 1986 May; 27(5): 486-96.
29. Novak M, Jakes R, Edwards PC, Milstein C, Wischik CM.: Proc Natl Acad Sci USA. 1991 Jul 1; 88(13): 5837-41.

Claims:

1. A DNA construct which comprises a cDNA molecule coding for N- and C-terminally truncated tau molecules, wherein
 - the molecules have truncated at least 30 nucleotides downstream of the start codon and truncated at least the 30 nucleotides upstream of the stop codon of the full length tau cDNA sequence coding for 4-repeat and 3-repeat tau protein, respectively, as given in Seq.accession number NM_173727 in GeneBank
 - the minimally truncated tau core encompasses a protein fragment which is encoded by nucleotides nr 744 - 930 (seq ID No. 9; numbered according to tau protein isoform 43)
 - said DNA constructs are coding for proteins, which have neurofibrillary (NF) pathology producing activity when expressed in brain cells of animals.
2. A transgenic non-human animal of whose germ and/or somatic cells comprises the DNA construct according to claim 1.
3. Non-human animal whose germ and somatic cells transiently or stably express said DNA construct according to claim 1, thereby exhibiting NF pathology in the brain.
4. A transgenic non-human animal according to claim 2 or 3, preferably a rat, wherein the protein encoded by said DNA molecules is expressed in the brain.
5. Methods for genotyping of transgenic animals of any one of claims 2 to 4 using oligonucleotides specific for transgenic truncated tau according to claim 1.
6. A transgenic animal according to claim 4, developing NF pathology, and having a genetic background allowing the induction of risk factors associated with AD, thereby representing a disease model for humans.
7. A transgenic non-human animal of claim 6, which represents an animal model of AD, and permits induction of hypertension as a risk factor of AD.

8. A transgenic non-human animal of claim 6, which represents an animal model of AD, and permits induction of diabetes as a risk factor of AD

9. A transgenic non-human animal of claim 6, which represents an animal model of AD, and permits induction of hypercholesterolemia as a risk factor of AD.

10. A screening assay system and validation system for substances for the treatment, prevention and diagnosis of Alzheimer's disease which comprises:

- evaluation of substances by:
 - detecting changes of neurofibrillar pathology in an animal according to any one of claims 2 to 4 and 6 to 9,
 - measuring of neurobehavioural changes in said animal,
 - measuring of the cognitive score in said animal,
- a validation system for substances for the treatment and prevention of tauopathies preferably AD,
- a validation system for the development of diagnostic markers and probes for the detection tauopathies preferably AD,
- a validation system for substances for the treatment of hypertension, diabetes, dislipidaemia and hypercholesterolemia in combination with tauopathies, preferably AD.

11. An experimental model system according to claim 10 for identifying new drug targets in tauopathies and related neurodegeneration processes preferably AD

12. Use of the animal according to any of claims 2 - 4 and 6 - 9 as an in-vivo assay to test the efficacy of substances, or therapies, in particular neurofibrillary pathology reducing therapies.

13. The use according to claim 12 wherein said substances or therapies are for neurodegenerative diseases, in particular tauopathies, preferably AD and other neurodegenerative diseases accompanied by neurofibrillary pathology.

14. A cell line transformed with a construct according to claim

1 or being derived from a transgenic animal of any one of claims 2 to 4 and 6 to 9.

15. A cell line according to claim 14, characterised in that the cell line is a rat cell line derived from a transgenic rat embryo.

16. An in vitro assay comprising a cell line according to claim 14 or 15, where said assay is employed as a screening and validation tool for the discovery of therapeutic preventive and diagnostic compounds and markers for Alzheimer's disease.

SEQUENCE LISTING

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<120> Transgenic Animal Expressing Alzheimer's Tau Protein

<130> R 41446

<150> A 1053/2002

<151> 2002-07-12

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INTERNATIONAL SEARCH REPORT

12 JAN 2004

International Application No
P 03/07390

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 A01K67/027 C12N5/06 G01N33/00 G01N33/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 53340 A (GURNEY MARK E ; ABRAHAM IRENE (US); UPJOHN CO (US)) 26 July 2001 (2001-07-26) the whole document	1-16
X	WO 01 65252 A (DUFF KAREN ; NATHAN S KLINE INST FOR PSYCHI (US)) 7 September 2001 (2001-09-07) see especially page 2, line 17 the whole document	1-16
X	WO 01 02552 A (DEN HAUTE CHRIS VAN ; GEERTS HUGO ALFONS GABRIEL (BE); JANSSEN PHAR) 11 January 2001 (2001-01-11) the whole document	1-16

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No.
EP 03/07390

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEWIS J ET AL: "Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 293, no. 5534, 2001, pages 1487-1491, XP002224859 ISSN: 0036-8075 the whole document	1-16
X	WO 96 30766 A (HOFFMANN LA ROCHE ;HARRINGTON CHARLES ROBERT (GB); WISCHIK CLAUDE) 3 October 1996 (1996-10-03) figure 22	1,14-16
X	NOVAK M ET AL: "MOLECULAR CHARACTERIZATION OF THE MINIMAL PROTEASE RESISTANT TAU UNIT OF THE ALZHEIMER'S DISEASE PAIRED HELICAL FILAMENT" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 12, no. 1, 1993, pages 365-370, XP009019837 ISSN: 0261-4189 the whole document	1,14-16
X	WO 01 18546 A (MANDELKOW ECKHARD ;MANDELKOW EVA MARIA (DE); MAX PLANCK GESELLSCHA) 15 March 2001 (2001-03-15) see especially the Sequence Listing the whole document	1,14-16

INTERNATIONAL SEARCH REPORT

International Application No

PC 03/07390

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0153340	A	26-07-2001	AU 2961901 A EP 1248518 A2 WO 0153340 A2 US 2002010947 A1	31-07-2001 16-10-2002 26-07-2001 24-01-2002
WO 0165252	A	07-09-2001	AU 4184901 A WO 0165252 A1 US 2003115621 A1	12-09-2001 07-09-2001 19-06-2003
WO 0102552	A	11-01-2001	AU 6267300 A CA 2372742 A1 WO 0102552 A2 EP 1216297 A2 JP 2003504015 T NO 20020010 A	22-01-2001 11-01-2001 11-01-2001 26-06-2002 04-02-2003 02-01-2002
WO 9630766	A	03-10-1996	AT 221662 T AU 5334496 A BR 9607846 A CA 2215397 A1 DE 69622701 D1 DE 69622701 T2 DK 817969 T3 WO 9630766 A1 EP 1067386 A2 EP 0817969 A1 ES 2180753 T3 JP 11502925 T PT 817969 T TR 9701039 T1 US 2002168687 A1 US 6376205 B1	15-08-2002 16-10-1996 14-07-1998 03-10-1996 05-09-2002 06-03-2003 25-11-2002 03-10-1996 10-01-2001 14-01-1998 16-02-2003 09-03-1999 31-12-2002 21-02-1998 14-11-2002 23-04-2002
WO 0118546	A	15-03-2001	AU 7653100 A CA 2384006 A1 WO 0118546 A2 EP 1214598 A2 JP 2003508788 T	10-04-2001 15-03-2001 15-03-2001 19-06-2002 04-03-2003

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